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Transcriptome analysis of high-temperature adult-plant resistance conditioned by *Yr39* during the wheat–*Puccinia striiformis* f. sp. *tritici* interaction

TRISTAN E. CORAM[1,2]*, MATTHEW L. SETTLES[3] AND XIANMING CHEN[1,2]

[1]*US Department of Agriculture, Agricultural Research Service, Wheat Genetics, Quality, Physiology and Disease Research Unit, Pullman, WA 99163, USA*
[2]*Department of Plant Pathology, and* [3]*Department of Molecular Biosciences, Washington State University, Pullman, WA 99164-6430, USA*

SUMMARY

Stripe rust [caused by *Puccinia striiformis* Westend. f. sp. *tritici* Eriks. (*Pst*)] is a destructive disease of wheat (*Triticum aestivum* L.) worldwide. High-temperature adult-plant (HTAP) resistance to stripe rust is race non-specific, inherited quantitatively and durable. Previously, we identified and mapped the single *Yr39* HTAP stripe rust resistance gene in the spring wheat cultivar Alpowa, which was identified on chromosome 7BL and accounted for 64.2% of the variation in resistance. To identify transcripts associated with *Yr39*-mediated resistance, we selected two F_7 recombinant inbred lines (RILs) from an 'Avocet S/Alpowa' cross that differed at the *Yr39* locus to represent an incompatible (*Yr39*) and compatible (*yr39*) interaction with *Pst*. Using the Affymetrix Wheat GeneChip, we profiled the transcript changes occurring in flag leaves of these two RILs over a time-course after treatment with *Pst* urediniospores and mock-inoculation. This time-course study identified 99 induced transcripts that were classified as HTAP resistance-specific. The temporal pattern of transcript accumulation showed a peak at 48 h after infection, which was supported by microscopic observation of fungal development and quantitative PCR assays that showed a rapid increase in fungal biomass after this time in the compatible interaction. More than half (50.5%) of the annotated transcripts specifically induced during HTAP resistance were involved in defence and/or signal transduction, including *R* gene homologues and transcripts associated with pathogenesis-related protein production, phenylpropanoid biosynthesis and protein kinase signalling. This study represents the first transcript profiling of HTAP resistance to stripe rust in wheat, and we compare our results with other transcript studies of race-specific and race non-specific resistance.

* *Correspondence*: Tel.: +1 509 335 1596; Fax: +1 509 335 2550;
E-mail: tcoram@mail.wsu.edu

INTRODUCTION

Stripe rust [caused by *Puccinia striiformis* Westend. f. sp. *tritici* Eriks. (*Pst*)] is an important and destructive disease of wheat (*Triticum aestivum* L.) worldwide (Chen, 2005). Genetic resistance is the preferred method for controlling stripe rust, of which two major types are race-specific and race non-specific resistance. Race-specific resistance to stripe rust includes the qualitatively inherited all-stage resistance, controlled by single major resistance (*R*) genes. Because of the specific nature of this resistance, all-stage *R* genes have been frequently overcome by new pathogen races (Chen, 2005). Conversely, high-temperature adult-plant (HTAP) resistance to stripe rust is race non-specific, inherited quantitatively and durable (Chen, 2005; Chen and Line, 1995a,b; Line, 2002; Line and Chen, 1995; Qayoum and Line, 1985). HTAP resistance usually varies from low to high levels and depends on plant growth stage, temperature and inoculum pressure. In contrast to all-stage *R* genes that are effective at overnight temperatures of 4 °C and day temperatures of 20 °C, HTAP resistance genes tend to be effective after stem elongation and when average overnight temperatures are > 10 °C and day temperatures are 25–30 °C (Line and Chen, 1995; Milus and Line, 1986a,b; Qayoum and Line, 1985). More than 30 genes conferring all-stage resistance to stripe rust have been identified (Chen, 2005; McIntosh *et al.*, 1998, 1999, 2001) in contrast to the relatively few identified HTAP resistance genes (Chen, 2005; Chen and Line, 1995a,b; Chicaiza *et al.*, 2006; Lin and Chen, 2007; Uauy *et al.*, 2005). Although HTAP resistance is often a polygenic trait, recent studies have shown that single genes can control a significant proportion of the variation in HTAP resistance (Bariana *et al.*, 2001; Börner *et al.*, 2000; Lin and Chen, 2007; Suenaga *et al.*, 2003; Uauy *et al.*, 2005).

Recently, we identified and mapped HTAP resistance to stripe rust in the spring wheat cultivar Alpowa (Chen *et al.*, 2003; Lin and Chen, 2007). Lin and Chen (2007) used the resistance gene analogue polymorphism (RGAP) approach to map the resistance in Alpowa, and identified a single major quantitative trait locus

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(QTL) that accounted for 64.2% of the variation in resistance to stripe rust. The QTL, which formed part of a linkage group on chromosome 7BL, showed single gene inheritance and was given the name *Yr39* (Lin and Chen, 2007). The level of resistance conferred by *Yr39* is similar to that of *Yr36*, which is also a major effect QTL located on chromosome 6BS (Uauy *et al.*, 2005). Currently, *Yr39* is the only HTAP resistance locus to be mapped on chromosome 7BL, and thus pyramiding *Yr39* with other sources of both race-specific and race non-specific resistance will be valuable.

Considering the agricultural value of *Yr39*, the objective of this study was to profile the transcript changes associated with *Yr39*-controlled resistance to stripe rust. Transcript profiling has the ability to reveal pathways of gene expression involved in a defence response, which may enable the elucidation of the overall defence mechanism controlled by *Yr39*. In wheat, this approach has recently been successful in dissecting race-specific resistance responses to stripe rust (Coram *et al.*, 2008), and adult plant resistance to leaf rust (Hulbert *et al.*, 2007). However, transcript profiling of HTAP resistance to stripe rust has not yet been performed. Subsequently, we selected two F_7 recombinant inbred lines (RILs) from an 'Avocet S'/'Alpowa' cross that differed at the *Yr39* locus to represent a resistant (*Yr39*) and susceptible (*yr39*) interaction with *Pst*. At the F_3 stage, both lines were selected against an additional resistance gene in Alpowa known as *YrAlp*, which is an all-stage resistance gene (Lin and Chen, 2007), so as not to confound the transcript changes associated with the adult plant phenotype. Additionally, to estimate background genetic differences not associated with *Yr39*, the two lines were surveyed using the wheat diversity array technology (DArT). Using the Affymetrix Wheat GeneChip, we profiled the transcription changes occurring in flag leaves of these two F_7 RILs over a time-course after treatment with *Pst* urediniospores and mock-inoculation. Time points for tissue sampling were chosen based on the expected timing of resistance expression after microscopic observation of fungal development and quantitative PCR estimation of fungal biomass accumulation.

RESULTS

Selection of plant material

The selected *Yr39* and *yr39* F_7 RILs for this study were determined to lack the *YrAlp* all-stage resistance phenotype and the *YrAlp* flanking RGAP markers *Xwgp47* and *Xwgp48* (Lin and Chen, 2007). The adult-plant phenotypes and genotypes of the two lines were also confirmed as *Yr39* and *yr39* by a greenhouse test and the *Yr39* flanking RGAP marker *Xwgp45* (Lin and Chen, 2007). The *Yr39* and *yr39* F_7 RILs were also selected based upon closest similarity across their genetic marker profile, which was previously assessed at the F_3 stage by Lin and Chen (2007). To obtain a further estimate of the background genetic difference between the two F_7 RILs we utilized the Wheat *PstI*(*TaqI*) v2.3 DArT (Triticarte, North Ryde, Australia). Comparison of the *Yr39* and *yr39* F_7 RIL DArT marker profiles revealed that, of the 642 markers with hybridization in at least one genotype, 229 (35.7%) were polymorphic (data not shown). In reference to the female parent (Avocet S), *Yr39* had 52.3% polymorphism and *yr39* had 51.2% polymorphism (data not shown); thus, the *Yr39* and *yr39* lines were more related to each other than to the female parent.

Fungal infection and disease development

Disease progression on both *Pst*-inoculated and mock-inoculated flag leaves of the *Yr39* and *yr39* F_7 RILs were monitored until 20 days post-inoculation (dpi). Flag leaves were chosen for inoculation and subsequent tissue collection as they possess greater resistance to *Pst* than other leaves (Chen, 2005). For both genotypes, flag leaves of all *Pst*-inoculated plants began to show chlorotic strips at ~7 dpi, which continued in the *yr39* genotype until ~14 dpi. However, in *Yr39* flag leaves, the chlorotic strips had become necrotic by 12–14 dpi, which was indicative of successful HTAP resistance. Heavy sporulation (formation of rust uredia) was observed on *Pst*-inoculated *yr39* flag leaves by ~15 dpi, which continued for the remainder of the 20-day observation period. The necrotic strips on the *Yr39* flag leaves remained until 20 dpi without any evidence of *Pst* pustule formation (data not shown). All mock-inoculated plants were free of any disease symptoms over the 20-day observation period.

To estimate the timing of *Pst* penetration and subsequent resistance expression, the development of fungal structures was examined using light microscopy over a time-course spanning 3–96 h post-inoculation (hpi). For both genotypes we found that spores began to germinate as early as 3 hpi, and most had germinated by 6 hpi. Germ tubes were also visible at 6–9 hpi, and initial stoma penetration, without appressoria formation, was observed at 9 hpi in both genotypes. By 12 hpi most germ tubes had penetrated stoma (Fig. 1), which continued over the following time-points from 24 to 96 hpi. The technique only allowed for visualization of structures on the outside of the flag leaves, so we could not observe subepidermal infection hyphae and haustoria formation. Overall, we saw no difference in the development of fungal structures between genotypes over the time-course.

To investigate the timing of resistance expression further, quantitative reverse-transcriptase PCR (qRT-PCR) was used to assess the abundance of the constitutively expressed *Pst* β-tubulin mRNA in reference to the wheat constitutively expressed elongation factor 1-alpha mRNA over the time-course (Fig. 2). Using *Pst* β-tubulin levels as an indicator of infection and fungal biomass, this assay revealed that similar levels of *Pst* were present in both the *Yr39* and the *yr39* interaction up to ~48 hpi. From

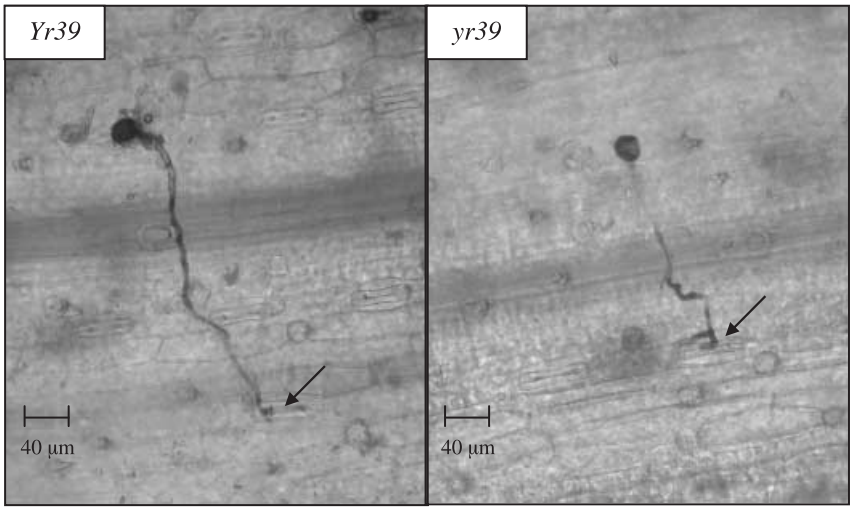

Fig. 1 *Puccinia striiformis* f. sp. *tritici* germ tubes penetrating stoma of flag leaves, as indicated by arrows, in both *Yr39* and *yr39* genotypes at 12 hpi.

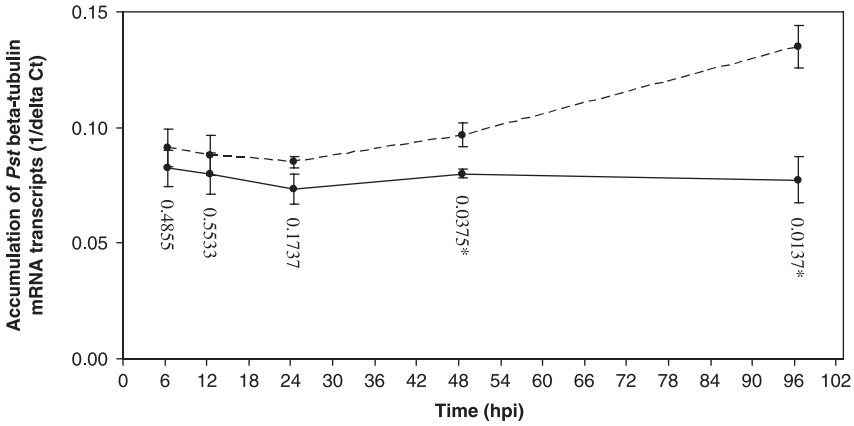

Fig. 2 Differential accumulation of *Puccinia striiformis* f. sp. *tritici* (*Pst*) β-tubulin mRNA transcripts in *Yr39* (solid line) and *yr39* (broken line) flag leaves after inoculation (mean ± standard error, $n = 3$). Reciprocal delta cycle threshold (Ct) values of *Pst* β-tubulin Ct values minus wheat elongation factor 1-alpha Ct values are plotted as determined by quantitative reverse-transcriptase PCR; hpi = hours post-inoculation. Student's *t*-test *P* values for the difference in mean are indicated for each time point, where those marked with an asterisk were considered significantly different at the 95% confidence level.

48 hpi onwards there was a significant ($P < 0.05$) increase of *Pst* in the *yr39* tissue compared with *Yr39*. This result indicated that *Pst* infection in *Yr39* was being restricted by 48 hpi, which we attribute to the expression of HTAP resistance.

GeneChip data quality

High-quality data were obtained from all 36 GeneChips as assessed by visual inspection of scanned images and the *affyQCreport* (Parman *et al*., 2005) of Bioconductor (Gentleman *et al*., 2004). For the Affymetrix recommended quality metrics, the ratio of largest to smallest average background (1.592), maximum to minimum scale factors (2.165), and largest to smallest percentage present (1.232) were all < 3.0, which indicates strong similarity across arrays. The ratio of hybridization efficiency at the 3′ and 5′ ends of the control probe sets was also < 3.0 as required for good quality data. The spiked-in hybridization controls from Affymetrix (BioB, BioC, BioD and CreX) also behaved as expected, which indicated good hybridization sensitivity (data not shown). Per array intensity distributions were conserved both before and after normalization, and correlation coefficients of summarized probe set data between replicates were all > 0.90. Finally, RNA degradation plots, normalized unscaled standard error (NUSE) plots, and relative log expression (RLE) plots indicated strong consistency among arrays (data not shown). A batch effect between array replications was removed before differential analysis using the 'ComBat' batch removal algorithm (Johnson *et al*., 2007). Subsequently, hierarchical clustering (Euclidean metrics, complete linkage) of arrays before and after batch removal showed that the algorithm successfully eliminated the batch effects without sacrificing the biological variation (supplementary Fig. S1).

Pst-induced gene expression changes

Measurements and comparisons of transcript abundance in *Pst*-inoculated and mock-inoculated *Yr39* and *yr39* F_7 RIL genotypes at 12, 24 and 48 hpi were performed using the wheat GeneChip,

and all data was analysed using Bioconductor packages (Gentleman *et al.*, 2004). All biological replications were pooled and *Pst*-inoculated data were compared with mock-inoculated data for each genotype to identify transcript differences attributable to *Pst*-inoculation. In order to focus on gene expression differences related to treatment, probe sets significant for the time effect were not analysed. Each probe set was functionally annotated using HarvEST (Affymetrix Wheat1 Chip version 1.52). Gene ontology (GO) was based on the TIGR rice genome annotation such that if a unigene possessed a significant (< 1e-10) BLASTx match to rice, as identified in HarvEST, the corresponding GO terms for the rice protein were used, if available. For the *Yr39* genotype, we identified 215 transcripts that were significantly differentially expressed according to treatment at the 48-hpi time-point (supplementary Table S1). No significant differential expression was detected at 12 or 24 hpi. Of the 215 transcripts, 207 were induced by *Pst*-inoculation and eight repressed. For *yr39*, significant differential expression was also not detected at 12 or 24 hpi, and just one transcript was significantly induced at 48 hpi.

The distribution of biological function for the 207 *Yr39 Pst*-induced transcripts included 90 (43.5%) transcripts putatively involved in pathogen defence-related pathways and signal transduction, including phenylpropanoid biosynthesis (anthocyanins and lignin), pathogenesis-related (PR) proteins and protein kinase proteins. A further 19 (9.2%) transcripts were involved in protein/carbohydrate transport, and 17 (8.2%) in metabolism, homeostasis, structure and/or cell death. Nine (4.3%) transcripts were involved in transcription regulation, four (1.9%) in energy production, 18 (8.7%) matched to hypothetical proteins and 50 (24.2%) possessed no homology. Of the eight *Yr39 Pst*-repressed transcripts, six were involved in energy production, one in metabolism and one possessed no homology. The single *yr39 Pst*-induced transcript possessed no homology, and was also induced in *Yr39*. Comparisons between GeneChip and quantitative RT-PCR (qRT-PCR) $\log_2$ expression ratios were made for 16 significant *Pst*-induced probe sets that were considered interesting because they were induced only in *Yr39* and were annotated as defence-related. All probe sets were validated with a PCR efficiency of 90–100%, and thus the results were compared without adjustment. Comparisons were made at 24 and 48 hpi, and the qRT-PCR fold change of each probe set was generally consistent with the GeneChip data (Table 1), which indicated that the GeneChip data was reliable.

HTAP resistance-specific transcripts

In attempt to identify transcripts specific to *Yr39*-controlled resistance, we examined the 215 *Yr39 Pst*-induced transcripts for significant genotype differences between *Yr39* and *yr39* at the 48-h time-point. *Yr39* data were compared with *yr39* data for each treatment to identify transcript differences attributable to genotype. Under mock-inoculation, 25 of the 215 *Yr39 Pst*-induced transcripts were significantly differentially expressed between genotypes, and thus could be attributed to background genetic differences (data not shown). Although these 25 transcripts may still be controlled by the *Yr39* locus, they were eliminated from this analysis as probably representing background genetic differences. Of the remaining 190 transcripts that were significantly induced in *Yr39 Pst*-inoculated samples compared with mock-inoculated samples, 99 were also significantly different between *Yr39* and *yr39 Pst*-inoculated samples (data not shown). Subsequently, in order to focus on transcripts most likely to be specific to *Yr39*-mediated resistance, these 99 transcripts were selected for analysis and considered as likely HTAP resistance-specific transcripts. The other 91 transcripts, although significantly induced in *Yr39 Pst*-inoculated samples compared with mock-inoculated samples, were not significantly different between the *Yr39* and *yr39 Pst*-inoculated samples, and thus were not identified as the best candidates for involvement in *Yr39*-mediated resistance.

All 99 HTAP resistance-specific transcripts were induced in response to *Pst*-inoculation, and comparison of the grouped mean $\log_2$ fold change (*Pst*-inoculated vs. mock-inoculated) for the 99 transcripts in *Yr39* and *yr39* over time showed that these transcripts were induced in both genotypes, but at a lower level in *yr39* (Fig. 3). Most of these transcripts (50.5%) were involved in pathogen defence-related pathways and signal transduction (Table 2). Of particular interest were nine *R* gene-like transcripts, including a homologue of the Yr10 all-stage stripe rust resistance protein (TaAffx.43336.1.S1_at), a Cf2/Cf5 disease resistance protein homologue (Ta.25518.1.S1_at) and three protein kinase transcripts with similarity to the barley stem rust resistance protein Rpg1 (Ta.10326.1.S1_a, Ta.10236.2.S1_a_at and Ta.10236.2.S1_x_at). Also of interest was a maize NADPH-dependent HC-toxin reductase Hm1 homologue (Ta.12946.1.S1_at), four transcripts of the wheat pathogen-induced WIR1A protein, five beta-1,3-glucanase transcripts and six phenylalanine ammonia-lyase (PAL) transcripts. Several protein kinase signalling proteins were identified, as well as transcription regulatory transcripts, including a WRKY5 homologue (TaAffx.80313.1.S1_at).

DISCUSSION

The Wheat GeneChip was used to profile the transcript accumulation patterns in two wheat F_7 RILs that differed for the presence of the agriculturally important *Yr39* stripe rust resistance gene. Transcript profiling of HTAP resistance to stripe rust has not yet been performed, and the comparison of GeneChip data within each RIL (*Pst*-inoculated vs. mock-inoculated) allowed for the examination of gene expression patterns involved in a resistant (*Yr39* race non-specific resistance) and susceptible interaction,

Table 1 Expression ratios for probe sets assessed by GeneChip (*Array*) and quantitative reverse-transcriptase PCR (*qPCR*).

			Mean $\log_2$ fold change			
			24 hpi		48 hpi	
Putative function	Probe set ID	Genotype	Array	qPCR	Array	qPCR
Protein kinase similar to barley stem rust resistance protein Rpg1	Ta.10236.2.S1_a_at	*Yr39*	0.04	0.22 ± 0.18	2.23*	1.19 ± 0.10
		yr39	–0.35	–0.33 ± 0.22	0.97	0.35 ± 0.27
Alternative oxidase	Ta.10549.2.A1_at	*Yr39*	0.66	0.32 ± 0.30	2.43*	1.53 ± 0.13
		yr39	–0.43	–0.68 ± 0.08	0.26	0.78 ± 0.22
Receptor-like protein kinase	Ta.11135.1.S1_at	*Yr39*	0.00	–0.52 ± 0.13	1.30*	0.91 ± 0.27
		yr39	–0.63	–0.55 ± 0.28	0.36	0.49 ± 0.19
NADPH-dependent HC-toxin reductase Hm1 homologue	Ta.12946.1.S1_at	*Yr39*	0.11	–0.11 ± 0.11	1.82*	1.46 ± 0.14
		yr39	–0.57	–0.63 ± 0.27	0.87	0.85 ± 0.16
Cf2/Cf5 disease resistance protein homologue	Ta.25518.1.S1_at	*Yr39*	–0.19	–0.23 ± 0.09	1.28*	1.09 ± 0.48
		yr39	–0.60	–1.24 ± 1.46	0.58	–0.05 ± 0.11
NBS-LRR disease resistance protein	Ta.25549.1.S1_at	*Yr39*	0.02	0.04 ± 0.19	1.44*	1.06 ± 0.14
		yr39	–0.27	–0.45 ± 0.11	0.34	0.32 ± 0.16
Leucine-rich repeat family protein	Ta.4479.2.S1_x_at	*Yr39*	–0.34	0.44 ± 0.25	1.05*	0.94 ± 0.21
		yr39	–0.62	–0.64 ± 0.20	0.43	0.54 ± 0.10
Pleiotropic drug resistance protein/ABC transporter	Ta.6990.1.S1_at	*Yr39*	0.25	0.57 ± 0.24	1.29*	1.02 ± 0.20
		yr39	0.02	0.17 ± 0.31	0.27	0.55 ± 0.25
Receptor-like protein kinase	Ta.7017.1.S1_at	*Yr39*	–0.09	–0.13 ± 0.09	1.51*	1.63 ± 0.12
		yr39	–0.43	NA	0.47	NA
Leucine-rich repeat transmembrane protein kinase	Ta.8590.1.S1_s_at	*Yr39*	0.15	–0.08 ± 0.20	1.29*	0.95 ± 0.20
		yr39	–0.21	–0.01 ± 0.20	0.50	0.27 ± 0.21
NB-ARC domain containing protein	TaAffx.103209.1.S1_at	*Yr39*	0.22	0.23 ± 0.29	1.36*	0.95 ± 0.21
		yr39	–0.15	–0.04 ± 0.12	0.30	0.37 ± 0.22
Receptor-like protein kinase	TaAffx.111955.1.S1_at	*Yr39*	–0.64	–0.64 ± 0.25	1.69*	1.25 ± 0.12
		yr39	–0.46	–0.31 ± 0.13	–0.03	0.18 ± 0.19
Heat-stress transcription factor	TaAffx.120360.1.A1_at	*Yr39*	0.22	0.22 ± 0.08	1.40*	1.70 ± 0.04
		yr39	–0.04	–0.07 ± 0.18	0.37	0.92 ± 0.04
CR4-NOT transcription complex subunit 8 protein	TaAffx.33753.1.S1_at	*Yr39*	0.46	0.50 ± 0.17	1.98*	1.36 ± 0.07
		yr39	–0.42	–0.37 ± 0.17	0.04	–0.21 ± 0.48
Putative stripe rust resistance protein Yr10	TaAffx.43336.1.S1_at	*Yr39*	–0.05	0.05 ± 0.11	1.25*	1.05 ± 0.28
		yr39	–0.25	–0.11 ± 0.22	0.99	1.09 ± 0.11
Putative WRKY5 protein	TaAffx.80313.1.S1_at	*Yr39*	0.06	–0.22 ± 0.20	1.02*	1.74 ± 0.03
		yr39	–0.33	–0.71 ± 0.37	0.48	0.66 ± 0.11

Array values indicate mean $\log_2$ fold change after *Puccinia striiformis* f. sp. *tritici*-inoculation, relative to the mock-inoculation data, for each genotype (standard error is not shown due to empirical Bayes variance correction during linear model analysis). qPCR values indicate $\log_2$ ratios of $2\hat{}(\Delta C_t\text{control}/\Delta C_t\text{treatment}) \pm$ standard error. Array values with an asterisk are those that were significant after linear model analysis ($P < 0.05$) with false discovery rate multiple testing correction ($\alpha < 0.05$) and fold change > 2.0 cutoff.

respectively. After the successful removal of a batch effect in the GeneChip data, significant differentially expressed transcripts were identified using linear models, where probability (*P*) values were adjusted for multiple comparisons using false discovery rate (FDR) $\alpha < 0.05$ (Benjamini and Hochberg, 1995) and transcript fold change > 2.0 (with respect to mock-inoculated controls). Correlation coefficients between biological replications were > 0.90 and comparisons between GeneChip and qRT-PCR results revealed common expression kinetics for most results, indicating the strong reliability of the GeneChip data. Because the exact timing of the *Yr39* race non-specific response after *Pst* infection was not known, we examined the development of fungal structures and accumulation of fungal biomass over time. These results indicated *Pst* penetration by 9–12 hpi, without appressoria formation as previously reported for stripe rust (Marryat, 1907; Moldenhauer *et al.*, 2006), and restriction of fungal spread by 48 hpi. Subsequently, the time-points chosen for microarray analysis (12, 24 and 48 hpi) were expected to encompass the gene expression changes associated with initiation of the resistance response in *Yr39*. The GeneChip results did confirm this expectation, where we observed significant induction of 99 putative HTAP resistance-specific transcripts at 48 hpi. Subsequently, this also provided some indication that the *Yr39*-mediated resistance to stripe rust is post-haustorial, but further studies would be required to confirm this.

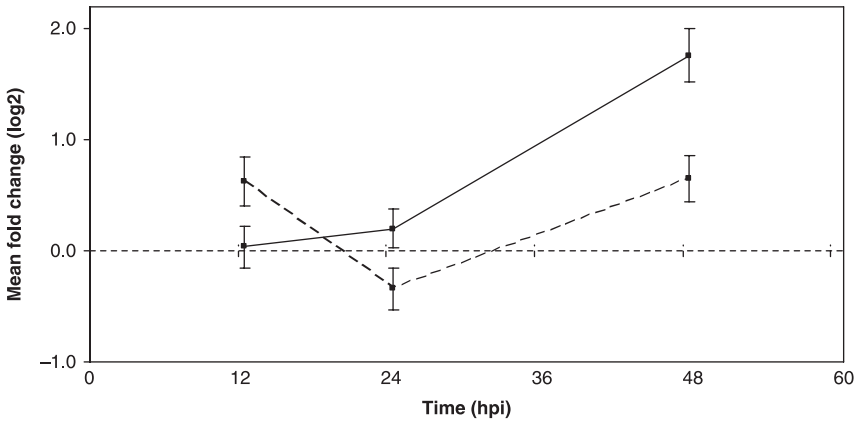

Fig. 3 Mean $\log_2$ fold change ± standard error of the 99 grouped high-temperature adult-plant (HTAP) resistance-specific transcripts for *Puccinia striiformis* f. sp. *tritici*-inoculated samples in reference to mock-inoculated samples for *Yr39* (solid line) and *yr39* (broken line) over the time course (12, 24 and 48 h post-inoculation).

Interestingly, we detected only one transcript (of unknown function) induced in the susceptible interaction (*yr39*), which may indicate that basal defences were not induced by 48 hpi. Also, six of the eight *Pst*-repressed transcripts in *Yr39* were involved in energy production, which may reflect a shift in cellular resources required for mounting a successful defence response. Direct comparison between the *Yr39* and *yr39* data allowed for the selection of HTAP resistance-specific transcripts that were probably not a result of background genetic differences between the genotypes. Additional transcripts significantly different between genotypes, but not induced within genotypes, were not focused upon considering their likelihood to represent background genetic differences not related to *Yr39*. The grouped mean $\log_2$ fold change (*Pst*-inoculated vs. mock-inoculated) of the HTAP-specific transcripts for each genotype showed that these transcripts were also induced in *yr39*, but not to a level of significance over the time course studied. It is possible that these transcripts may become significantly induced in *yr39* at time points later than 48 hpi, which is a delay that may contribute to lack of resistance. We did select the *Yr39* and *yr39* F_7 RILs to be closely related based on previous F_3 genetic marker data, but background genetic differences were detected using the wheat DArT, where the *Yr39* and *yr39* lines were found to possess 35.7% polymorphism but were more closely related to each other than to the female parent (Avocet S). However, the proportion of polymorphic DArT markers may inflate genetic differences as these markers are pre-screened to be highly polymorphic. In fact, in a separate DArT study, we found two BC_7:F_4 near-isogenic lines to possess 22.0% polymorphism (unpublished data). Subsequently, our selection of *Yr39* and *yr39* lines did narrow genetic variation to a degree, although not to a level of near-isogenicity that could have been achieved by selecting a single F_7 RIL segregating at the *Yr39* locus and generating F_7-derived F_9 homozygous lines.

In an attempt to separate race-specific and race non-specific responses to stripe rust in wheat, we compared the results with those obtained in our previous study of *Yr5*-mediated race-specific resistance (Coram *et al.*, 2008), which allowed a comparison of the functional categories represented by the two different resistance mechanisms (Table 3). The biological pathways represented by both resistances were substantially conserved. The key findings were that race non-specific resistance involved more diverse induction of defence-related pathways, including the induction of several R protein homologues, wider induction of the phenylpropanoid pathway, and several other putative defence transcripts. By contrast, race-specific resistance showed higher levels of PR protein induction, as well as hypersensitive-response transcripts. Thus, we postulate that the durability of *Yr39*-mediated race non-specific resistance lies in the ability to induce a broad defence response that is not directed toward a specialized response. In wheat, results such as this have also been observed in race non-specific resistance to leaf rust controlled by the *Lr34* gene, where wide-ranging defensive pathways were induced (Hulbert *et al.*, 2007).

Putative biological function of HTAP resistance-specific transcripts

The first step in effective disease resistance is host recognition of the pathogen. For typical race-specific resistance, the mechanism is controlled by gene-for-gene interaction between the host *R* gene and pathogen avirulence (*Avr*) gene. However, race non-specific resistance genes, such as the *mlo* powdery mildew resistance gene of barley, are thought to be more durable because they are not dependent on the recognition of a single *Avr* gene (Piffanelli *et al.*, 2002; Wolter *et al.*, 1993). For *Yr39*-mediated race non-specific resistance to stripe rust, we found that nine R protein-like transcripts were specifically induced in the resistance response (Table 2). This observation may reflect the non-specific nature of HTAP resistance for recognizing multiple Avr proteins, which may explain its durability. Furthermore, the induction of the R protein-like transcripts may be associated with the necrotic (hypersensitive) reactions observed in HTAP resistance. Among

Table 2 List of the 99 transcripts considered as *Yr39*-controlled high-temperature adult-plant resistance-specific transcripts significantly induced by *Puccinia striiformis* f. sp. *tritici* (*Pst*) in reference to mock-inoculated controls at 48 h post-inoculation.

Functional category	Putative function	Probe set ID	Log_2 FC	*P* value
Cell death	Senescence-associated protein	Ta.14231.2.S1_x_at	1.79	0.025
Defence	Putative disease resistance protein	Ta.14786.1.S1_at	3.06	0.019
Defence	Putative disease resistance protein	Ta.22482.1.S1_s_at	1.60	0.040
Defence	Pleiotropic drug resistance protein/ABC transporter	Ta.6990.1.S1_at	1.29	0.014
Defence	Putative latex protein allergen	Ta.9588.2.S1_a_at	2.25	0.013
Defence—alkaloid	Strictosidine synthase	TaAffx.56754.1.S1_at	1.11	0.037
Defence—anthocyanin	Hydroxyanthranilate hydroxycinnamoyl transferase	Ta.14063.1.S1_at	1.35	0.014
Defence—anthocyanin	UDP-glucosyl transferase	Ta.8495.1.A1_at	3.05	0.037
Defence—anthocyanin	UDP-glucosyl transferase	TaAffx.23237.1.S1_at	1.74	0.014
Defence—cell wall	Pathogen induced WIR1A protein	Ta.22732.1.S1_s_at	2.27	0.010
Defence—cell wall	Pathogen induced WIR1A protein	Ta.22732.1.S1_x_at	1.85	0.014
Defence—cell wall	Pathogen induced WIR1A protein	Ta.3133.1.S1_x_at	2.33	0.005
Defence—cell wall	Pathogen induced WIR1A protein	Ta.97.2.S1_x_at	2.13	0.005
Defence—detoxification	NADPH-dependent HC-toxin reductase Hm1 homologue	Ta.12946.1.S1_at	1.82	0.035
Defence—oxidative stress	Peroxidase	Ta.21307.1.S1_x_at	2.70	0.023
Defence—oxidative stress	Germin-like protein	TaAffx.15880.1.S1_at	1.56	0.027
Defence—phenylpropanoid	Phenylalanine-ammonia lyase	Ta.20429.1.S1_at	2.19	0.044
Defence—phenylpropanoid	Phenylalanine-ammonia lyase	Ta.28046.1.A1_at	1.92	0.005
Defence—phenylpropanoid	Phenylalanine-ammonia lyase	Ta.7022.1.S1_at	1.97	0.005
Defence—phenylpropanoid	Phenylalanine-ammonia lyase	Ta.7022.1.S1_s_at	3.10	0.001
Defence—phenylpropanoid	Phenylalanine-ammonia lyase	Ta.7022.1.S1_x_at	1.93	0.005
Defence—phenylpropanoid	Phenylalanine-ammonia lyase	TaAffx.92008.1.A1_s_at	1.73	0.021
Defence—PR protein	Beta-1,3-glucanase	Ta.1174.1.S1_x_at	2.80	0.027
Defence—PR protein	Beta-1,3-glucanase	Ta.21354.1.A1_at	1.83	0.010
Defence—PR protein	Beta-1,3-glucanase	Ta.21354.1.A1_x_at	1.84	0.016
Defence—PR protein	Beta-1,3-glucanase	Ta.22427.1.A1_x_at	2.45	0.007
Defence—PR protein	Beta-1,3-glucanase	Ta.26048.1.S1_x_at	1.57	0.025
Defence—R protein	Protein kinase—similar to barley stem rust R protein Rpg1	Ta.10236.2.S1_a_at	2.23	0.005
Defence—R protein	Protein kinase—similar to barley stem rust R protein Rpg1	Ta.10236.2.S1_x_at	1.74	0.014
Defence—R protein	Protein kinase—similar to barley stem rust R protein Rpg1	Ta.10326.1.S1_at	1.89	0.043
Defence—R protein	Cf2/Cf5 disease resistance protein homologue	Ta.25518.1.S1_at	1.28	0.005
Defence—R protein	NBS-LRR disease resistance protein	Ta.25549.1.S1_at	1.44	0.010
Defence—R protein	Leucine-rich repeat family protein	Ta.4479.2.S1_x_at	1.05	0.025
Defence—R protein	Leucine-rich repeat transmembrane protein kinase	Ta.8590.1.S1_s_at	1.29	0.037
Defence—R protein	NB-ARC domain containing protein	TaAffx.103209.1.S1_at	1.36	0.033
Defence—R protein	Putative stripe rust resistance protein Yr10	TaAffx.43336.1.S1_at	1.25	0.035
Defence—signalling	Protein kinase	Ta.10236.1.A1_at	1.86	0.014
Defence—signalling	Receptor-like protein kinase	Ta.11135.1.S1_at	1.30	0.019
Defence—signalling	Protein kinase	Ta.12007.2.S1_at	1.47	0.037
Defence—signalling	Mitogen-activated protein kinase	Ta.236.1.S1_at	1.27	0.024
Defence—signalling	Receptor-like protein kinase	Ta.7017.1.S1_at	1.51	0.049
Defence—signalling	Serine/threonine protein kinase	Ta.7718.2.S1_a_at	1.16	0.036
Defence—signalling	Receptor-like protein kinase	TaAffx.111955.1.S1_at	1.69	0.009
Defence—stress-induced	Alternative oxidase	Ta.10549.2.A1_at	2.43	0.037
Defence—stress-induced	Alternative oxidase	Ta.28112.1.S1_at	1.94	0.043
Defence—stress-induced	Cold-acclimation induced protein	Ta.351.2.S1_x_at	1.48	0.022
Defence—stress-induced	ERD1 protein—water-stress induced	Ta.4014.2.S1_at	1.43	0.010
Energy—electron transport	Blue copper-binding protein	Ta.18203.1.S1_at	1.74	0.043
Energy—electron transport	Blue copper-binding protein	Ta.5654.1.S1_at	1.67	0.024
Energy—electron transport	Blue copper-binding protein	TaAffx.55612.1.S1_at	1.50	0.037
Metabolism	Shikimate kinase	Ta.8618.1.S1_at	1.58	0.020
Metabolism	Prephenate dehydratase	Ta.9122.1.S1_x_at	1.20	0.037
Metabolism	Glucosyl hydrolase	TaAffx.9022.1.S1_at	2.24	0.035
Metabolism—proteolysis	Aspartyl protease	Ta.15123.1.A1_at	1.08	0.027

Table 2 *Continued.*

Functional category	Putative function	Probe set ID	Log_2 FC	*P* value
Metabolism—proteolysis	Protein phosphatase	TaAffx.16090.1.S1_at	1.67	0.026
Signal transduction	Calmodulin-binding heat shock protein	Ta.10168.1.S1_at	1.07	0.037
Signal transduction	Calmodulin-binding protein	Ta.7711.1.A1_at	2.00	0.007
Signal transduction	Ankyrin-like protein	TaAffx.12271.1.S1_at	1.44	0.005
Signal transduction	Calmodulin-like protein	TaAffx.128621.1.S1_at	1.81	0.005
Transcription	Heat-stress transcription factor	TaAffx.120360.1.A1_at	1.40	0.024
Transcription	CR4-NOT transcription complex subunit 8 protein	TaAffx.33753.1.S1_at	1.98	0.027
Transcription	Putative WRKY5 protein	TaAffx.80313.1.S1_at	1.02	0.014
Transport	Phosphate transporter	Ta.10084.1.S1_at	1.32	0.023
Transport	Histidine amino acid transporter	Ta.12339.1.S1_s_at	1.04	0.025
Transport	Monosaccharide transport protein	Ta.12517.1.S1_at	1.90	0.026
Transport	Integral membrane protein	Ta.15082.1.S1_at	1.76	0.024
Transport	Integral membrane protein	Ta.15082.1.S1_x_at	1.65	0.016
Transport	Putative membrane protein	Ta.23392.1.S1_at	2.27	0.023
Transport	Ammonium transporter	Ta.27506.1.S1_at	1.99	0.037
Transport	Histidine amino acid transporter	Ta.28479.1.S1_at	1.69	0.017
Transport	Integral membrane protein	Ta.29523.1.S1_at	1.91	0.009
Transport	Histidine amino acid transporter	Ta.3869.1.S1_at	2.27	0.027
Transport	UDP-galactose transporter	Ta.4921.1.S1_at	1.69	0.014
Transport	Potassium transporter	Ta.9064.2.S1_s_at	1.33	0.031
Transport	Integral membrane protein	TaAffx.52897.1.S1_at	1.95	0.021
Transport	ATPase	TaAffx.54526.1.S1_at	2.32	0.007
Unknown	Hypothetical protein	Ta.14129.1.S1_at	1.96	0.024
Unknown	Hypothetical protein	Ta.22669.1.A1_at	1.89	0.041
Unknown	Hypothetical protein	Ta.6155.2.S1_a_at	1.14	0.007
Unknown	Hypothetical protein	Ta.954.1.S1_s_at	1.23	0.034
Unknown	No homology	Ta.13991.1.S1_x_at	1.92	0.022
Unknown	No homology	Ta.20149.1.S1_at	2.01	0.023
Unknown	No homology	Ta.21531.1.S1_at	1.43	0.014
Unknown	No homology	Ta.24564.1.S1_a_at	1.28	0.005
Unknown	No homology	Ta.24564.1.S1_x_at	1.39	0.005
Unknown	No homology	Ta.24564.3.S1_x_at	1.17	0.005
Unknown	No homology	Ta.29984.1.S1_at	2.36	0.005
Unknown	No homology	Ta.30753.1.A1_at	1.32	0.040
Unknown	No homology	Ta.520.1.S1_at	1.57	0.036
Unknown	No homology	Ta.5518.1.S1_at	2.85	0.005
Unknown	No homology	TaAffx.108203.1.S1_at	1.87	0.033
Unknown	No homology	TaAffx.110215.2.S1_at	2.57	0.001
Unknown	No homology	TaAffx.2056.1.A1_at	2.03	0.029
Unknown	No homology	TaAffx.27427.1.S1_at	1.00	0.044
Unknown	No homology	TaAffx.27956.1.S1_at	1.47	0.027
Unknown	No homology	TaAffx.55533.1.S1_at	1.37	0.037
Unknown	No homology	TaAffx.56501.1.S1_at	2.05	0.005
Unknown	No homology	TaAffx.7236.1.S1_at	2.13	0.027
Unknown	No homology	TaAffx.84007.1.S1_at	1.62	0.024

Significant transcripts possessed a log_2 fold change (log_2 FC) > 1.0 and were significant after linear model analysis ($P < 0.05$) with false discovery rate multiple testing correction ($\alpha < 0.05$). *P* value indicates significance after multiple testing correction. Functional categories were based on the Munich Information Center for Protein Sequences classifications and putative function shows the best significant BLASTX database hit from HarvEST.

the nine R protein-like transcripts, we identified a homologue of the *Yr10* wheat stripe rust race-specific resistance gene. *Yr10*, originally identified in Turkish line P.I.178383, represents the only sequenced stripe rust resistance gene from wheat so far (GenBank AF149114). The gene is located on chromosome 1B (Chen *et al.*, 1995) and encodes a leucine-zipper NBS-LRR protein. Virulence against *Yr10* has been found in the United States (Chen, 2005), and thus the *Yr10* homologue induced

Table 3 Comparison of the functional biological categories and selected transcripts induced during *Yr5*-mediated all-stage race-specific resistance (Coram *et al.*, 2008) and *Yr39*-mediated race non-specific resistance to stripe rust.

Functional category	*Yr5* race-specific resistance	*Yr39* race non-specific resistance
Defence		
R proteins		
— Yr10 homologue	–	+
— Rpg1 homologue	–	+
— Cf2/Cf9 homologue	–	+
— Hm1 homologue	–	+
Cell wall		
— WIR1A protein	+	+
— Proline-rich protein	+	–
PR proteins		
— Beta-1,3-glucanase	+	+
— PR protein 10	+	–
— Thaumatin-like protein	+	–
— Chitinase	+	–
Phenylpropanoid		
— PAL	+	+
— UDP-glucosyl transferase	–	+
— Hydroxyanthranilate hydroxyl cinnamoyl transferase	–	+
Oxidative stress		
— Peroxidase	+	+
Hypersensitive response		
— S1/P1 nuclease	+	–
— Chromosome condensation factor	+	–
Miscellaneous		
— Pleiotropic drug resistance/ABC transporter	–	+
— Putative disease resistance protein	–	+
— Latex protein allergen	–	+
Signal transduction		
— Protein kinase	+	+
— Receptor protein kinase	–	+
— Calmodulin protein	+	+
Transcription		
— WRKY5 homologue	–	+
— Myb transcription factor	+	–
Transport		
— Carbohydrate	+	+
— Amino acid/protein	–	+
— Ammonium/phosphate/potassium	–	+
Electron transport		
— Cytochrome P450	+	–
— Blue copper-binding protein	+	+

during *Yr39*-mediated resistance may only partially contribute to the race non-specific resistance controlled by the *Yr39* gene on chromosome 7BL. The qPCR results also showed that the *Yr10* homologue was induced during the compatible interaction, which indicates that it is not responsible for resistance on its own. Other transcripts were specifically induced during *Yr39*-mediated resistance (confirmed by qPCR), and included homologues of the Rpg1 barley stem rust receptor kinase resistance protein, the Cf2/Cf5 LRR protein for *Cladosporium fulvum* resistance in tomato, and other putative R proteins (Table 2). Additionally, the wheat probe set Ta.12946.1.S1_at, with homology to the Hm1 NADPH-dependent HC-toxin reductase protein for *Cochliobolus carbonum* resistance in maize, was also specifically induced in *Yr39*. The diversity of induced R protein-like homologues may demonstrate the non-specific nature of *Yr39*-mediated resistance, which includes the control of various *R* gene transcripts that, when taken together, can confer effective resistance. Importantly, our previous study of *Yr5*-mediated race-specific resistance found no evidence for the induction of other R protein-like transcripts, which may be an important

difference between race-specific and race non-specific resistance.

The obvious effect of increased R protein-like transcript expression would be increased cellular signalling involved in typical *R*-gene-mediated pathways. We did observe this effect through the identification of 14 HTAP resistance-specific transcripts associated with signal transduction and/or transcription control (Table 2). Several of these encoded protein kinases, including mitogen-activated protein kinase (MAPK) and receptor-like kinases, which are implicated in signal transduction events that regulate defence responses following *R*-gene-mediated pathogen perception (Garcia-Brugger *et al.*, 2006). In particular, MAPK cascades are thought to control the expression of WRKY transcription factors, which play an important role in mediating defence responses (Euglem, 2006; Ross *et al.*, 2007). To support this, we also identified one induced HTAP resistance-specific WRKY transcription factor transcript. Other transcript profiling studies have also reported the induction of protein kinases and WRKY transcription factors, including the barley response to *Fusarium graminearum* infection (Boddu *et al.*, 2006) and the soybean response to *Phakopsora pachyrhizi* (Van de Mortel *et al.*, 2007). In wheat, the study of race non-specific resistance to leaf rust controlled by *Lr34* identified significantly induced MAPKs and WRKYs (Hulbert *et al.*, 2007), and our previous study of *Yr5*-mediated race-specific resistance also found evidence for protein kinase signalling cascades and transcription factor induction (Coram *et al.*, 2008), which would be expected given that *Yr5* behaves like a typical *R* gene.

There is evidence that WRKY transcription factors promote the expression of PR proteins (Ross *et al.*, 2007), which may explain our identification of five HTAP resistance-specific beta-1,3-glucanase (PR-2) transcripts. PR proteins are widely induced during host responses to pathogens, including basal defence, race-specific resistance and race non-specific resistance. In the present study, several PR proteins were induced in the incompatible interaction but the five beta-1,3-glucanse transcripts were classified as HTAP resistance-specific. Several PR proteins were implicated in our previous study of *Yr5*-mediated race-specific resistance (Coram *et al.*, 2008), and the transcript study of race non-specific leaf rust resistance in wheat performed by Hulbert *et al.* (2007) also identified higher levels of PR protein expression during the incompatible interaction. During *Yr39*-mediated resistance we also observed specific induction of the WIR1A protein, which is involved in cell wall defence. In wheat, the WIR1 protein functions by increasing adhesion of the cellular membrane to the cell wall during pathogen attack (Bull *et al.*, 1992). In barley, WIR1 homologues were induced during both a basal defence and a race-specific resistance response to powdery mildew (*Blumeria graminis* f. sp. *hordei*) infection (Jansen *et al.*, 2005). A WIR1 homologue was also reported to be induced in barley in response to barley leaf rust (*Puccinia hordei*) and the non-host wheat leaf rust pathogen (*Puccinia triticina*), where the authors concluded that WIR1 contributes to pre-haustorial resistance against leaf rusts and belongs to 'a battery of basic defence genes induced upon rust pathogen attack' (Neu *et al.*, 2003). Further, Marcel *et al.* (2007) recently identified a WIR1 homologue coincident with a QTL for barley leaf rust resistance. This apparent association of the WIR1 protein with non-specific resistance to rusts supports our finding that WIR1 is involved in race non-specific resistance to stripe rust in wheat. Hulbert *et al.* (2007) also found induction of WIR1 homologues in wheat during both race non-specific resistance and basal defence to leaf rust. However, as we also observed, expression levels for these transcripts were higher in the resistant genotype than in the susceptible interaction. Taken together, these data suggest that WIR1 is induced non-specifically during both host and non-host resistance, and is involved in conferring *Yr39*-mediated resistance through higher induction compared with the compatible interaction.

Other HTAP resistance-specific transcripts that warrant discussion include the pleiotropic drug resistance protein/ABC transporter homologue, which are known to be involved in detoxification (Jasinski *et al.*, 2003). Boddu *et al.* (2006) identified ABC transporters as important for detoxification of trichothecene during barley resistance to *Fusarium graminearum*, although the rust fungi are not thought to secrete toxins. Subsequently, the induction of an ABC transporter transcript, as well as the Hm1 homologue described above, may reflect the broad and non-specific nature of the *Yr39*-mediated response. The six HTAP resistance-specific phenylalanine-ammonia lyase (PAL) transcripts (Table 2) indicate increased activity of the phenylpropanoid pathway during race non-specific resistance. PAL is a central enzyme in the phenylpropanoid pathway, controlling the biosynthesis of defensive compounds such as anthocyanins, lignin and phytoalexins (Dixon *et al.*, 2002). Several PAL transcripts were also significantly induced during race non-specific *Lr34*-mediated resistance to leaf rust in wheat (Hulbert *et al.*, 2007), including three of the same transcripts found in this study (Ta.20429.1.S1_at, Ta.28046.1.A1_at and TaAffx.92008.1.A1_s_at). Subsequently, over-expression on the phenylpropanoid pathway may be common to race non-specific resistance to rusts in wheat. Interestingly, we previously identified PAL transcripts in *Yr5*-mediated race-specific resistance and basal defence, which indicates a broad role for phenylpropanoids in pathogen responses. Another overlap with the *Lr34* study of Hulbert *at al.* (2007) was the high level of peroxidase induction during race non-specific resistance, which suggests that an oxidative burst response occurs.

CONCLUSION

This study represents the first transcript profiling study of race non-specific, HTAP resistance against stripe rust in wheat. Race non-specific resistance to stripe rust is agriculturally valuable

because of its durability, and thus the understanding of the resistance mechanism will be important. This study was carefully designed to capture the transcript response occurring during HTAP resistance, through the selection of sampling time-points based on microscopic observation and quantitative estimation of fungal development. Subsequently, the expression of *Yr39*-mediated resistance was found to be post-haustorial, and involved the induction of a wide range of known defence mechanisms. In comparison with race-specific resistance and other race non-specific resistance studies, we conclude that *Yr39*-mediated race non-specific resistance involves a greater diversity of defence-related transcript induction, with particular emphasis on the induction of numerous *R* gene homologues that may control increased signalling and expression of defence-related products. This conclusion bears similarity to that of Ellis *et al.* (2007), who postulated that the difference between basal and *R*-gene-dependent defence is 'simply the level of expression of the same pathway'. Overall, the results of this study have identified transcripts associated with HTAP resistance to stripe rust in wheat, which will be utilized in further functional studies to identify causal genes involved in race non-specific resistance.

EXPERIMENTAL PROCEDURES

Selection of plant material and fungal isolate

The spring wheat cultivar Alpowa possesses the *Yr39* HTAP resistance, and two F_7 RILs from a cross with spring wheat 'Avocet Susceptible' (AVS*Alpowa:F_7) were selected as the *Yr39* and *yr39* genotypes for this study. The lines were derived from a population generated by Lin and Chen (2007), who determined both the *YrAlp* and the *Yr39* genotype and phenotype for 136 F_3 lines. At the F_7 stage, absence of the *YrAlp* all-stage resistance phenotype was confirmed by a compatible interaction with *Pst* race PST-21 in a greenhouse test, which was performed according to Lin and Chen (2007). DNA was then extracted from tissue of both F_7 RILs using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Using the DNA samples, the absence of the *YrAlp* genotype was confirmed with the flanking resistance gene analogue polymorphism (RGAP) markers *Xwgp47* and *Xwgp48*, according to the method of Lin and Chen (2007). The adult-plant resistance phenotypes of the two F_7 RILs were also confirmed as *Yr39* and *yr39* by a greenhouse test with *Pst* race PST-78 (performed as described belo under Experimental design). Disease reactions were scored at 14 days post-inoculation (dpi). The *Yr39* and *yr39* genotypes were determined in the F_7 RILs with RGAP marker *Xwgp45* (Lin and Chen, 2007). A pure isolate of *Pst* race PST-78 (Chen, 2005), which was avirulent on *Yr39* and virulent on *yr39* (like any other *Pst* races), was selected for this study and maintained on susceptible genotypes.

In selecting the *Yr39* and *yr39* genotypes for this study, similarity across their genetic marker profile as previously assessed by Lin and Chen (2007) at the F_3 stage was also considered, except for the *YrAlp* and *Yr39* markers. To obtain a further estimate of the background genetic differences between the two F_7 RILs we utilized the Wheat *PstI*(*TaqI*) v2.3 Diversity Array Technology (DArT) (Triticarte, North Ryde, Australia), which surveyed 986 highly polymorphic loci as biallelic dominant markers across the wheat genome (for wheat DArT details refer to Akbari *et al.*, 2006). DNA samples of both the *Yr39* and the *yr39* F_7 RILs, as well as the female parent Avocet S, were sent to Triticarte for processing in duplicate, and DArT scores were provided after analysis with DArTsoft™ software (Triticarte). Each DArT marker was scored as 0 (absent), 1 (present) or X (missing data). For each marker, Q values estimated marker quality, where Q values < 77 were regarded as unreliable data (E. Huttner, personal communication).

Experimental design

For each of three biological replications, *Yr39* and *yr39* F_7 RIL seeds were planted in 6-inch round pots using a potting mix (6 peat moss: 4 vermiculite with lime: 3 sand: 3 commercial potting mix: 2 perlite: 1.7 g/L lime: 3.3 g/L Osmocote: 2.2 g/L ammonium nitrate). For each genotype in each biological replication, eight pots were planted with three seedlings in each, where groups of two pots were randomly assigned to one of three harvest times (12, 24 and 48 h post-inoculation). Seedlings from the remaining group of pots were used to monitor the expected disease responses to inoculation. Seedlings were grown to the flag leaf stage with ligule emerged (Feekes stage 9.0, ~35 days) in a greenhouse with a stable temperature of 25 ± 2 °C and a 16-h light/8-h dark cycle. Inoculation was performed by misting the plants with sterile water and applying a 1 : 20 pure urediniospore/talc mixture to leaves, paying particular attention to inoculate the flag leaves uniformly. Talc was used to aid in the spread and adhesion of spores over leaf surfaces. Mock-inoculated plants were treated the same way except for the absence of spores in the talc. All treatments for each biological replication were performed at 09:00 h Pacific Standard Time. To promote spore germination, all plants were transferred to a dew chamber (100% relative himidity) operating at 10 °C in the dark for 24 h, before being placed in a growth chamber with a diurnal temperature cycle of 10 °C (02:00 h) to 35 °C (14:00 h) and a 16-h light/8-h dark cycle, which was required for HTAP resistance. All flag leaves from tillers of individual plants were harvested from groups of pots at the assigned times for RNA extraction.

Microscopic observation of infection process

The infection stages of *Pst* race PST-78 in both the *Yr39* and the *yr39* F_7 RILs were monitored using light microscopy observation.

Eight pots of each genotype, containing three seedlings each, were cultivated and inoculated as described in the Experimental design. Each pot was assigned one of seven harvest times (3, 6, 9, 12, 24, 48 and 96 h post-inoculation), and the remaining pot of each genotype was used to monitor the expected disease responses to inoculation. Flag leaves harvested at each time-point were cleared and fungal structures stained according to technique #3 of Liberato *et al.* (2005). Briefly, flag leaves were immersed in a mixture of 100% acetic acid and absolute ethanol (1 : 1) in a 50-mL tube, and incubated in a 60 °C water bath for 1 h. Cleared leaves were then rinsed in distilled water and fungal structures were stained by mounting on a microscope slide in 85% lactic acid with 1 g/L aniline blue. Leaf sections were examined using an Olympus BH-2 compound microscope (100–400× magnification).

Wheat GeneChip probe array

The GeneChip® Wheat Genome Array (Affymetrix, Santa Clara, CA) is a 3′IVT array that includes 61 127 probe sets representing 55 052 transcripts for all 21 wheat chromosomes in the genome. Of these probe sets, 59 356 represent modern hexaploid (A, B and D genomes) bread wheat (*T. aestivum*) and are derived from the public content of the *T. aestivum* UniGene Build #38 (24 April 2004); 1215 probe sets are derived from ESTs of a diploid near relative of the A genome (*T. monococcum*), a further 539 represent ESTs of the tetraploid (A and B genomes) macaroni wheat species *T. turgidum*, and five are from ESTs of a diploid near relative of the D genome known as *Aegilops tauschii*. Probe sets consisted of pairs of 11 perfect match (PM) and mismatch (MM) 25-mer oligonucleotides designed from the 3′ end of exemplar sequences, with nucleotide 13 as the MM. Array annotation information is available on the NetAffx data analysis centre (http://www.affymetrix.com).

Target synthesis and GeneChip hybridization

Flag leaves from the six seedlings corresponding to each experimental condition were quickly cut, pooled and frozen in liquid nitrogen. Total RNA was extracted from 1.0 g of pooled tissue using the Trizol® Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) with an on-column DNase treatment. Purified total RNA samples were quantified with a NanoDrop® ND-1000 (NanoDrop, Wilmington, DE) spectrophotometer, and satisfactory purity was indicated by $A_{260:280}$ ratios of 1.9–2.1 in 10 mM Tris-Hcl (pH 7.5). Integrity of total RNA samples was assessed by denaturing formaldehyde gel electrophoresis, where the presence of sharp 28S and 18S ribosomal RNA bands at an intensity ratio of ~2 : 1 (28S : 18S) indicated good integrity. Labelled probes were prepared using GeneChip one-cycle target labelling and control reagents according to the manufacturer's protocol (Affymetrix). Ten micrograms of labelled cRNA was used for each hybridization. All hybridizations and data acquisition was performed at the Bioinformatics Core Facility at Washington State University according to standard Affymetrix protocols (http://www.bioinformatics.wsu.edu).

Data analysis

GeneChip data analysis was performed at the Bioinformatics Core Facility. Firstly, using GeneChip Operating Software (GCOS) v.1.4 (Affymetrix), image quality control was performed by inspecting raw intensity (DAT) files for scratches/smears and uniform performance of the B2 oligo around the border of each image. Data quality control was assessed using the *affyQCreport* (Parman *et al.*, 2005) of Bioconductor (Gentleman *et al.*, 2004), which provided Affymetrix recommended quality metrics, per array intensity distributions, between array comparisons, and other diagnostic plots for each of the 36 GeneChip hybridizations. The Bioconductor (Gentleman *et al.*, 2004) package *affy* (Gautier *et al.*, 2004) was used to read in the 36 raw Affymetrix '.CEL' files, which were pre-processed using Robust Multi-array Average (RMA) (Bolstad *et al.*, 2003; Irizarry *et al.*, 2003). Hierarchical clustering (Euclidean metrics, complete linkage) of normalized arrays using the *hclust* function in R (R Development Core Team, 2006) revealed a batch effect between replications, where replicates hybridized and scanned on different days did not cluster together as expected. To remove this effect before differential analysis, the 'ComBat' batch removal algorithm (Johnson *et al.*, 2007) was implemented, which is an empirical Bayes framework that adjusts data for non-biological variation. The data were then separated into the three time-points and a linear model (Smyth, 2005) was applied on each probe set to detect significantly ($P < 0.05$) different transcript levels for genotype, treatment and the genotype × treatment interaction. Probability (P) values were adjusted for multiple comparisons using false discovery rate (FDR) $\alpha < 0.05$ (Benjamini and Hochberg, 1995). Additionally, for all tests, differentially expressed probe sets must have possessed a fold change > 2.0 to be considered. All minimum information about microarray experiments (MIAME) guidelines were observed and GeneChip data were deposited into WheatPLEX (Shen *et al.*, 2005), under accession number TA11.

Differentially expressed probe sets were identified and annotated using HarvEST (Affymetrix Wheat1 Chip version 1.52), which identified the corresponding unigene for each probe set and provided the current best BLASTX hit from the non-redundant (nr) database of NCBI, as well as the best BLASTX hits from the rice and *Arabidopsis thaliana* TIGR databases (http://www.tigr.org/plantProjects.shtml). A database hit < 1e-10 was considered as significant, otherwise the unigene was annotated as 'no homology'. For gene ontology (GO), the rice locus matching each probe set in the HarvEST output was queried using the TIGR rice genome annotation (Yuan *et al.*, 2003), which provided GO terms including biological function. Unigenes were assigned to functional

categories based on Munich Information Center for Protein Sequences (MIPS; http://mips.gsf.de/projects/funcat) classifications.

Quantitative RT-PCR

Sixteen target probe sets were selected for confirmation of GeneChip expression ratios by quantitative RT-PCR. The sequence of the unigene corresponding to each probe set was identified in HarvEST. Forward and reverse primers for quantitative detection were designed using PrimerQuest (Integrated DNA Technologies, http://www.idtdna.com) with the 'real-time' parameter set. For each genotype, treatment and time-point, 1.0 μg total RNA from one biological replication used for GeneChip hybridization was converted to cDNA template using random hexamers and iScript reverse transcriptase (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The resulting cDNA products were diluted to 200 μL in sterile water. An elongation factor 1-alpha unigene from Affymetrix probe set Ta.659.1.S1_at, whose expression remained consistent among all experimental conditions, was selected as the normalization gene for relative quantification of the target probe sets. Before proceeding with quantitative PCR, validation of each primer pair was performed according to Coram *et al*. (2008). To pass validation, each primer pair must have demonstrated 90–100% amplification efficiency. Triplicate quantitative RT-PCRs were then performed on experimental samples using iQ Sybr Green Supermix (Bio-Rad) with primers (400 nM each) and 5 μL of cDNA. Control reactions containing untranscribed RNA confirmed that no interfering genomic DNA products were present. PCR was performed on a Bio-Rad iQ5 Real-Time PCR Detection System instrument (Bio-Rad) with the following cycling programme: 95 °C for 3 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s 72 °C. All products were subjected to melting curve analysis and verified by gel electrophoresis. Relative fold changes were calculated by the comparative C_T method ($\Delta\Delta C_T$ method).

Fungal biomass and growth was assessed by quantification of the constitutively expressed *Pst* β-tubulin (Ling *et al*., 2007) mRNA in reference to the wheat constitutively expressed elongation factor 1-alpha mRNA in both *Yr39* and *yr39 Pst*-inoculated flag leaves over a time course (6, 12, 24, 48, 96 hpi). RNA was extracted, converted to cDNA and quantitative RT-PCR carried out as described above. *Pst* β-tubulin C_T values were normalized by subtracting the wheat elongation factor 1-alpha C_T values (delta C_T).

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Fig. S1 Hierarchical clustering (Euclidean metrics, complete linkage) of GeneChip data before (left) and after (right) the removal of a batch effect using the 'ComBat' algorithm.

Table S1 List of the transcripts significantly induced and repressed by *Puccinia striiformis* f. sp. *tritici* (*Pst*) in reference to mock-inoculated controls during *Yr39*-mediated high-temperature adult-plant resistance at 48 h post-inoculation. Significant transcripts possessed a $\log_2$ fold change ($\log_2$ FC) > 1.0 and were significant after linear model analysis ($P < 0.05$) with false discovery rate multiple testing correction ($\alpha < 0.05$). Functional categories were based on the Munich Information Center for Protein Sequences classifications and putative function shows the best significant BLASTX database hit from HarvEST.

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